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Note d'application

Development of Simple, Fast SPE Protocols for Basic Analyte Extraction with Phospholipid Removal Using Oasis PRiME MCX

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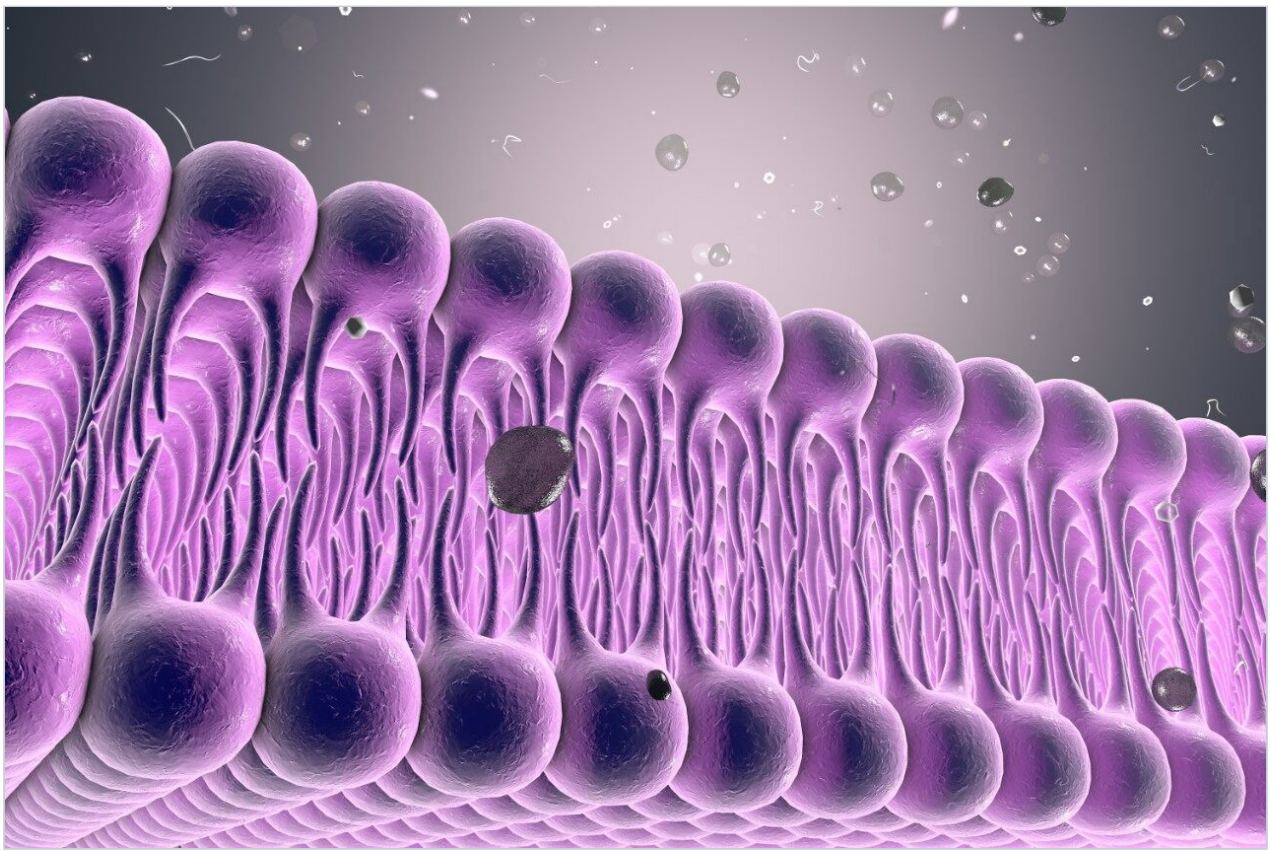

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Abstract

This application note highlights improved SPE methods for extracting basic analytes from blood plasma using Oasis PRiME MCX sample preparation products. The new Oasis PRiME MCX Protocols give greatly reduced concentrations of phospholipids compared to the Oasis MCX 2 x 4 Protocol 1, while maintaining high and reproducible recoveries for analytes with pK_a values greater than 5.

Benefits

- Improved removal of phospholipids from blood plasma using mixed-mode RP/SCX SPE
- Reduced matrix effects in LC-MS analyses of basic analytes
- Increased LC-MS system operating time and prolonged column life due to reduction in fouling

Introduction

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is one of the most widely used techniques employed to quantify analytes in biological samples. Endogenous phospholipids in such samples complicate the analysis. Phospholipids cause powerful matrix effects[1] where the analyte MS signals are suppressed or enhanced. This causes variation in analyte peak areas and therefore the reported analyte concentrations when those peaks overlap the region where the phospholipids elute. Phospholipids can also strongly adhere to the LC stationary phase.[2] Their presence on the chromatographic column can change the selectivity and retention of the analytes. Eventually, the column becomes saturated with phospholipids and the excess bleeds off continuously. This causes matrix effects, regardless of the analyte elution time. This is most problematic with the short narrow columns favored by analysts seeking rapid LC-MS/MS cycle times with maximum sensitivity. Furthermore, the LC-MS system can become fouled if phospholipids build up in the interface.

A variety of sample preparation approaches have been developed to overcome these challenges. Protein precipitation with a solvent such as acetonitrile is commonly used, but is not effective at removing phospholipids.[3] Solid-phase extraction (SPE) techniques work better, but vary in their effectiveness for removing phospholipids.[2,3,4] When analyzing basic compounds, a mixed-mode reversed-phase/strong cation-exchange (RP/SCX) SPE sorbent can selectively retain and concentrate such analytes. The proper

choice of load and wash conditions is key to the successful removal of phospholipids while maintaining high analyte recoveries. This note describes the development of simple, rapid protocols for this separation using SPE with Oasis PRiME MCX, a new family of mixed-mode RP/SCX sample preparation products based on Oasis MCX Technology.

Experimental

Sample 1: Phosphatidylcholine (PC) standard

L-α-phosphatidylcholine (100 mg, Sigma #P3556) was dissolved in methanol (1 mL). Acetonitrile (80 mL) and water (19 mL) were then added. The mixture was gently warmed briefly to obtain a homogeneous solution. The cartridge (1 cc/30 mg) SPE protocols described herein were performed on this solution. To the eluates from the Elute step, 35 µL of 98% formic acid was added to neutralize the ammonium hydroxide, thus avoiding hydrolysis of the PC. An aliquot of 35 µL of 98% formic acid was also added to the eluates of each Load, Aqueous Wash, and Organic Wash step to maintain the same volume. The collected eluates were vortexed to mix prior to injection.

Instruments:	ACQUITY UPLC I-Class with PDA Detector
Data management:	Empower 3 CDS

LC conditions

Column:	ACQUITY UPLC BEH C_8 1.7 µm 2.1 × 50 mm (p/n: 186002877)
Column temp.:	60 °C
Injection volume:	1.4 µL

Flow rate:	0.5 mL/min
Mobile phase A:	100% water
Mobile phase B:	100% acetonitrile
Gradient:	0.1% B for 0.5 min
	0.1 to 60% B over 6.5 min
	60 to 95% B over 1 min
	95% B for 1.5 min
	95 to 0.1% B over 0.3 min
	0.1% B for 0.7 min
	(Total time: 10.5 min)
Detection:	UV at 200 nm

Sample 2: Plasma with basic analytes

Rat blood plasma (Bioreclamation IVT #RATPLEDTA2) and a mixture of the basic analytes listed in Table 1 were subjected to the 96-well plate (10 mg sorbent per well) SPE protocols described herein. The previously described pre-spike and post-spike procedure was followed.[3] Specifically, during the Load step with each SPE protocol, four sorbent wells received plasma plus analytes (pre-spike) and four sorbent wells received only blank plasma. During the Elute step, the eluate for the blank plasma wells then received the analytes (post-spike). A final Elute step eluate concentration of 31.3 ng/well was achieved for both the pre-spike and post-spike wells. The ratio of each analyte multiple reaction monitoring (MRM) response in the averaged pre-spike vs. post-spike Elute step samples provided analyte % recovery for each SPE protocol. The total averaged phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) MRM peak areas in the Elute step allowed the comparison of the residual phospholipid concentrations from the different SPE protocols.

Instruments:	ACQUITY UPLC I-Class with Xevo TQ-S Mass Spectrometer (MS/MS)
Data management:	MassLynx v4.1

Method conditions

Column:	ACQUITY UPLC BEH C_{18} 1.7 µm, 2.1 x 100 mm (p/n: 186002352)
Column temp.:	40 °C
Injection volume:	2.0 µL
Flow rate:	0.6 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient:	2% B for 1 min
	2 to 70% B over 3 min
	70 to 95% B over 0.1 min
	95% B for 2.9 min
	95 to 2% B over 0.5 min
	2% B for 0.5 min

	(Total Time: 8.0 min)
Detection:	ESI Positive MRM (MS/MS Multiple Reaction Monitoring)
See Tables 1 and 2.	
Cone voltage:	30 V
Capillary voltage:	1.5 kV
Cone gas flow:	150 L/hr
Desolvation gas flow:	1000 L/hr
Nebulizer (bar):	7
Desolvation temp.:	500 °C

Compound I.D.	Compound name	Compound pK_a (ref. 5)	Precursor ion (M+H)	Product ion mass (M+H)	Collision voltage
1	Caffeine	10.4	195.16	106.04	30
2	Oxymorphone	8.17	302.17	198.24	44
3	Procainamide	9.32	236.10	120.25	30
4	Methamphetamine	9.87	150.24	91.04	15
5	Oxycodone	8.53	316.10	241.08	30
6	Codeine	8.21	300.20	165.10	30
7	MDMA	10.38	194.21	105.18	22
8	Clonidine	8.05	230.09	159.94	30
9	Diazepam	3.4	285.07	154.94	30
10	Risperidone	8.76	411.32	110.17	50
11	Propranolol	9.42	260.26	116.23	18
12	Protriptyline	8.2	264.27	155.19	20
13	Amitriptyline	9.4	278.23	91.19	24
14	Verapamil	8.92	455.44	150.17	44
15	Phencyclidine (PCP)	8.29	244.17	86.02	10

Table 1. Basic analytes: MRM transitions/conditions and pKa values.

Phospholipid I.D.*	Precursor ion (M+H)	Product ion mass (M+H)	Collision voltage (V)
LPC 16:0	496.40	184.10	30
LPC 18:2	520.40	184.10	30
LPC 18:1	522.40	184.10	30
LPC 18:0	524.40	184.10	30
PC 30:1	704.40	184.10	30
PC 34:2	758.40	184.10	30
PC 34:1	760.40	184.10	30
PC 36:3	784.40	184.10	30
PC 36:2	786.40	184.10	30
PC 38:6	806.40	184.10	30
PC 38:5	808.40	184.10	30

*Table 2. PC and LPC class phospholipids: MRM transitions/conditions. *LPC is lysophosphatidylcholine and PC is phosphatidylcholine. The first value is the total number of carbon atoms in the tail(s) and the second value is the number of double bonds in the tail(s).*

Results and Discussion

Background

The major class of phospholipids found in blood plasma is phosphatidylcholine (PC), making up approximately 70% of total human plasma phospholipids. Lysophosphatidylcholine (LPC) makes up approximately 10% of total human plasma phospholipids.[6] As shown in Figure 1, these phospholipids have two regions: a polar head and one or two non-polar tails. The polar head contains one ionized group, the positively charged quaternary amine, and one ionizable moiety, the negatively chargeable phosphate. The polar head is therefore very hydrophilic, being surrounded by a water solvation layer in aqueous solutions. The non-polar tails can be any of a number of long chain saturated or unsaturated fatty acid esters. This creates a very hydrophobic region. In aqueous solutions, PC forms bilayers to minimize interactions of the tails with water. The presence of these very different regions makes phospholipid removal challenging.

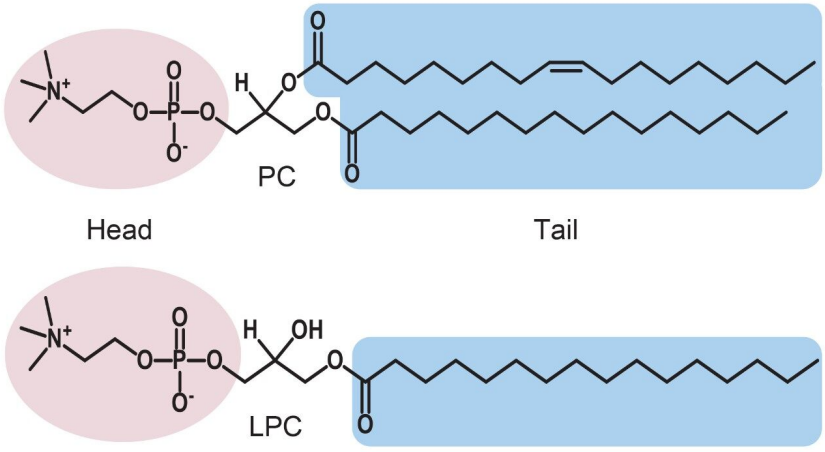

Figure 1. Representative structures of a Phosphatidylcholine, PC, and a Lysophosphatidylcholine, LPC.

With mixed-mode RP/SCX SPE sorbents such as Oasis MCX,[7] the phospholipid tail can have only hydrophobic contacts with hydrocarbon moieties in the sorbent. The polar head can interact in two ways. The water solvated head can participate in hydrogen bonding with the polar groups in the sorbent. Also, the positively ionized quaternary ammonium and the negatively ionizable phosphate groups may allow ionic interactions with the sorbent. The phosphate group in PC has been reported to have a pK_a of 2.6.[8] At a solution pH above approximately 2.6, the head group is predominantly zwitterionic and therefore has no net charge.[9] However, as the pH is lowered below approximately 2.6, an increasing fraction of the phosphate groups become neutralized and the phospholipid head becomes progressively more net positively charged. SPE sorbents with SCX functionality have sulfonic acid groups as the strong cation-exchange component. Such groups have a very low pK_a of <0.5. It is therefore possible that PC and LPC, under acidic conditions, can undergo cation-exchange on an SCX type sorbent, as shown in Figure 2.

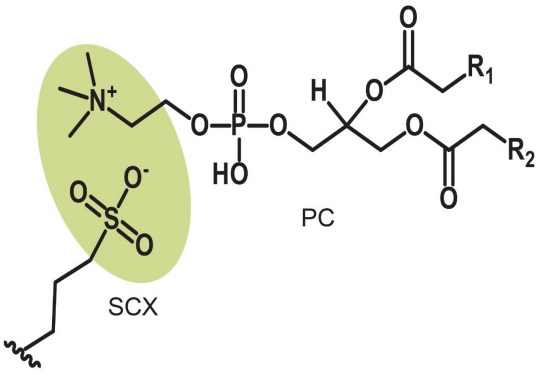

Figure 2. Possible cation exchange of the PC head on an SCX sorbent.

Current mixed-mode RP/SCX SPE protocols for the purification of basic analytes involve loading the sample under acidic conditions (e.g. dilute phosphoric acid). This ensures that the basic analytes are retained by cation exchange in addition to reversed-phase interactions. The sorbent is then washed with an acidic aqueous solution to remove polar interferences, then with an organic solvent to remove non-polar interferences. Finally, the analytes are eluted using a solution of a strong base in an organic solvent. This is the foundation of the Oasis MCX 2 x 4 Protocol 1 for bases.[10] If PC and LPC are held on the sorbent only by hydrophobic interactions, they should be removed in the Organic Wash step. However, it has been reported that very large wash volumes are needed to remove these phospholipids.[4] With typical wash volumes, significant concentrations of PC and LPC are found in the final eluate when using this method.[2,3,4] This is consistent with a combination of cation-exchange and hydrophobic mechanisms keeping these phospholipids on the sorbent until the Elute step with such SPE protocols.

Development of an improved SPE protocol for phospholipid removal

To investigate improvements in the removal of phospholipids in mixed-mode RP/SCX SPE, we used a phosphatidylcholine standard dissolved in acetonitrile/methanol/water (80/1/19 v/v/v). The fraction of PC obtained from each step in the SPE method was determined using UPLC with UV detection. Using the Oasis MCX 2 x 4 Protocol 1 for SPE cartridges, Figure 3, the majority of the PC (85%) was present in the Elute step. Very little PC (<3%) was found in the Aqueous Wash and Organic Wash steps, while a greater amount (12%) was found in the Load step. Table 3 summarizes these findings.

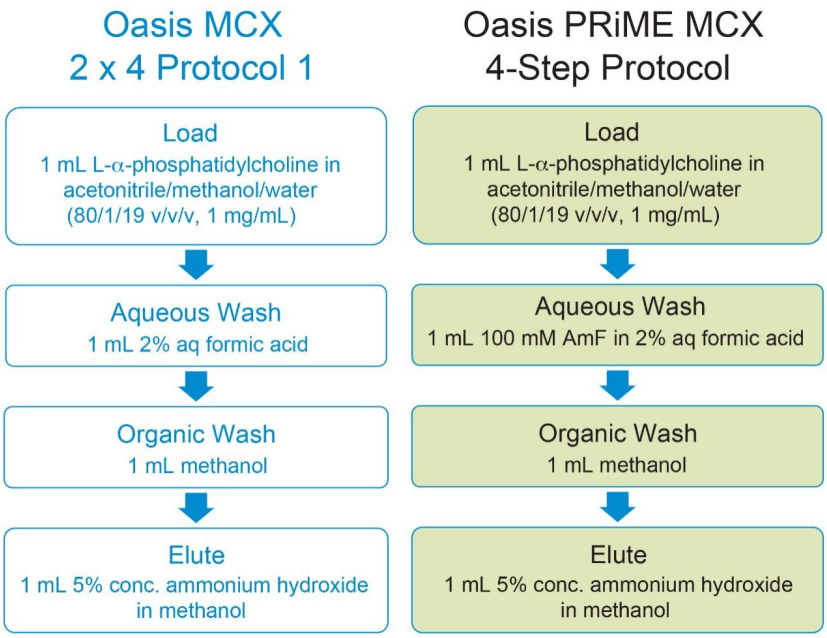

Figure 3. SPE protocols used for 1 cc/30 mg Oasis MCX Cartridges and Oasis PRiME MCX Cartridges.

Fraction	Oasis MCX 2 x 4 Protocol 1	Oasis PRiME MCX 4-step Protocol
Load	11.9% ± 1.1%	10.3% ± 1.1%
Aqueous Wash	0.3% ± 0.1%	0.2% ± 0.1%
Organic Wash	3.3% ± 0.6%	88.7% ± 1.3%
Elute	84.5% ± 1.3%	0.8% ± 0.1%

Table 3. PC amounts (% of total) found in each step of two different SPE protocols.

We investigated a number of changes to the SPE method, focused on removing more of the phosphatidylcholine in the Organic Wash step. Little improvement was found by using different solvents and solvent mixtures. However, when 100 mM ammonium formate (AmF) in methanol was used in the Organic Wash step, virtually all of the PC was removed. This is consistent with retention of the PC by both ionic and hydrophobic interactions on this sorbent. Both interactions must be disrupted for effective removal. Unfortunately, the analytes were also removed in this step.

A modification of this method selectively removed the phosphatidylcholine while most basic analytes were retained until the Elute Step. In this optimized method, called the Oasis PRiME MCX 4-step Protocol, the

Aqueous Wash step contains 100 mM ammonium formate. The results obtained for the phosphatidylcholine standard, using this method, show only approximately 0.8% of the PC in the Elute step. This is a 100-fold reduction versus the Oasis MCX 2 x 4 Protocol 1. These results are summarized in Table 3 and illustrated by the chromatogram in Figure 4. Most of the PC (89%) was removed in the Organic Wash step. With this optimized 4-step Protocol, very high analyte recoveries (>90%) were found in the Elute step.

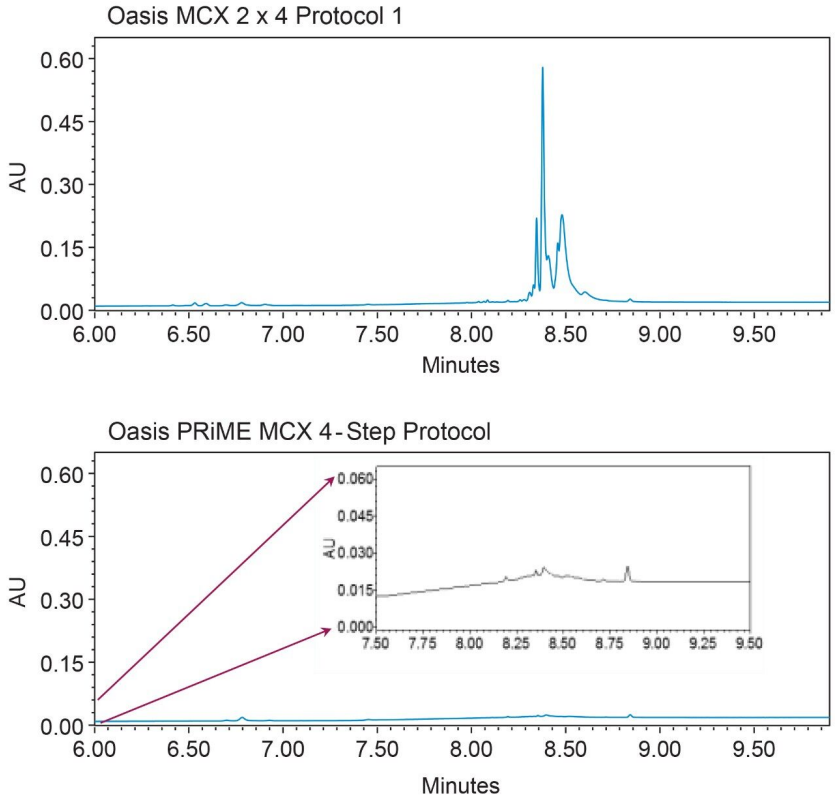

Figure 4. UPLC chromatograms showing the PC levels from the Elute step for a PC standard using the Oasis MCX 2 x 4 Protocol 1 and the Oasis PRiME MCX 4-step Protocol.

Extraction of basic analytes from plasma using the oasis prime MCX 4-step Protocol

The Oasis PRiME MCX 4-step Protocol was evaluated vs the Oasis MCX 2 x 4 Protocol 1 for the extraction of a panel of basic analytes spiked into rat plasma. The SPE methods used for the Oasis PRiME MCX 96-Well Plate (10 mg/well) (p/n: 186008915) are shown in Figure 5. The concentrations of phospholipids in the eluates from the elute step were determined using tandem mass spectrometry (MS/MS) following a previously described method.[11] Analyte recoveries were determined by comparing the MS peak areas obtained from samples spiked before extraction vs samples spiked post-extraction.[10] Fifteen analytes were

studied. The plasma samples were diluted 1:1 v/v with aq 4% phosphoric acid before loading. The results, Figure 6, show that the Oasis PRiME MCX 4-step Protocol gives an approximately 10-fold decrease in the phospholipids amount in the Elute step compared to the Oasis MCX 2 x 4 Protocol 1. The two methods gave similar high recoveries, Figure 7, except for Diazepam. Unlike the other analytes, Diazepam is a weak base (pK_a 3.4), and was found to be removed in the Aqueous Wash step of the Oasis PRiME MCX 4-step Protocol which has a pH of about 2.8. These results show that for a range of basic analytes with pK_a values greater than 5, the Oasis PRiME MCX 4-step Protocol gives high recoveries with an approximately 10-fold reduction in the concentration of phospholipids.

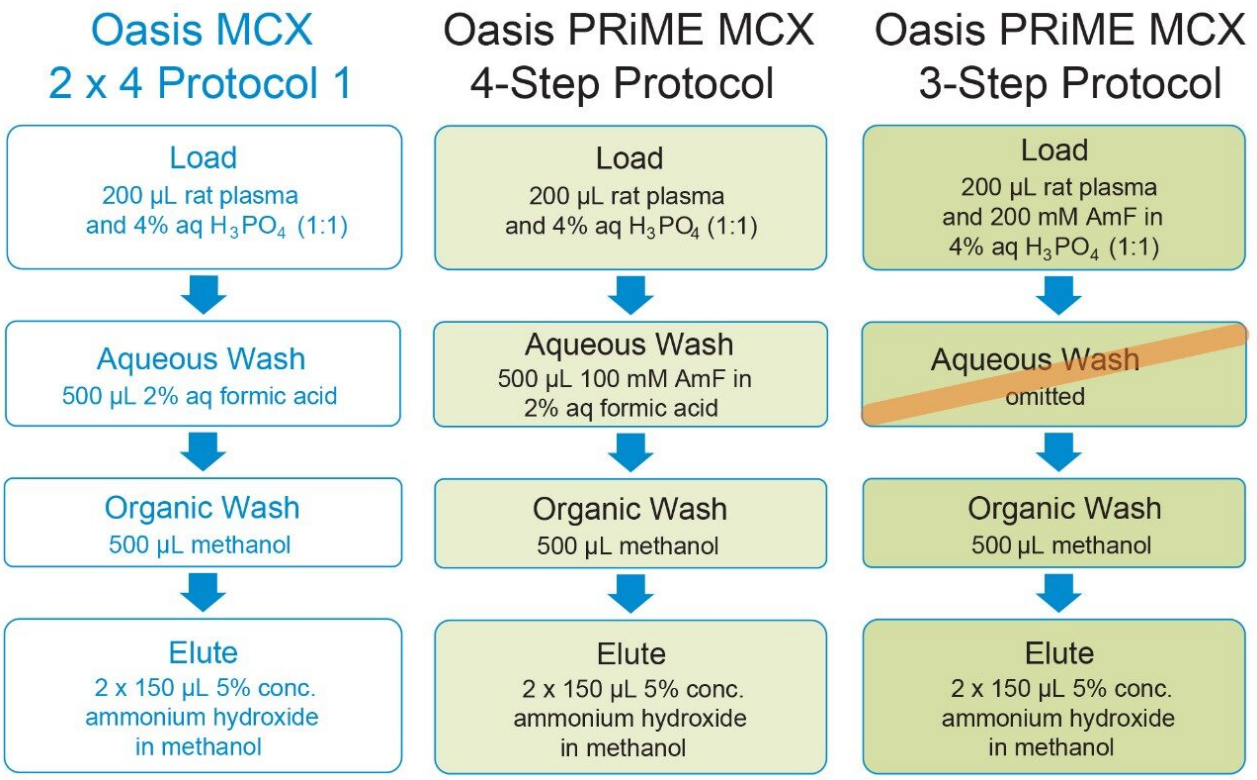

Figure 5. SPE protocols used for Oasis MCX and Oasis PRiME MCX 96-Well Plates (10 mg/well); the Oasis PRiME MCX protocols are patent pending.

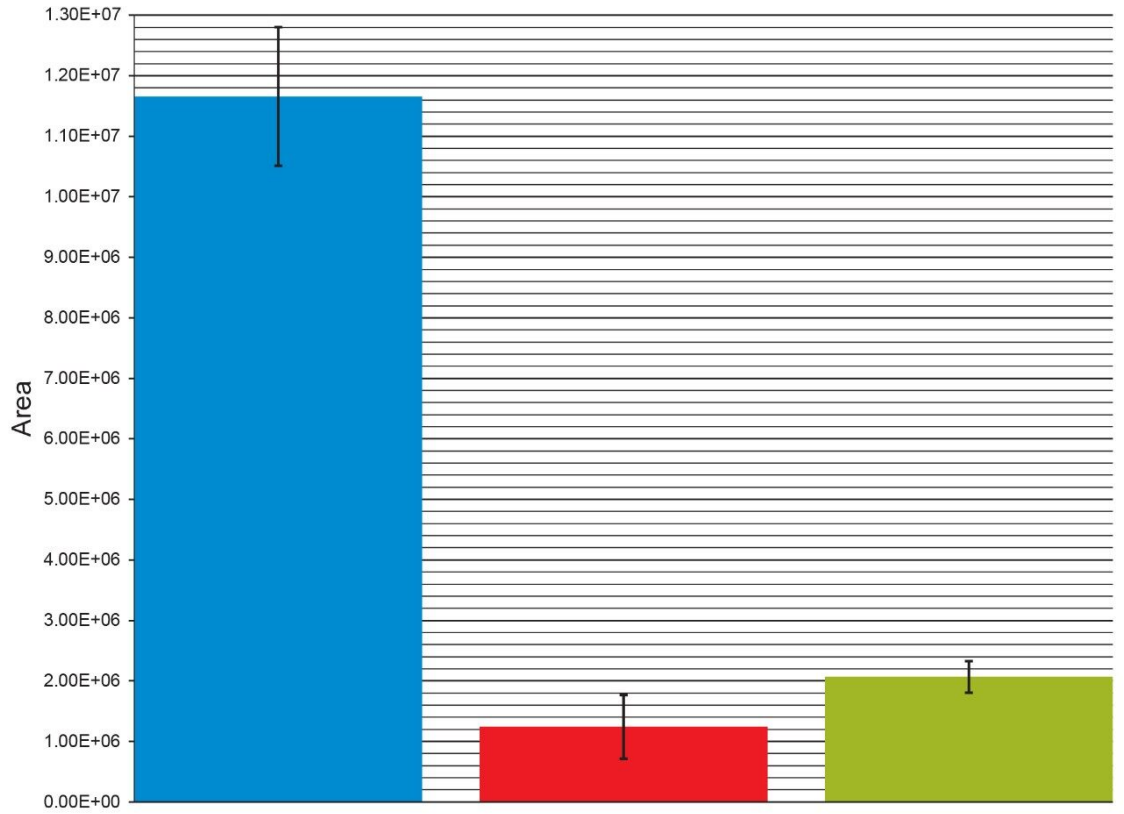

Figure 6.. Elute step phospholipid peak area from rat plasma using the Oasis MCX 2 x 4 Protocol 1 (blue, left), the Oasis PRiME MCX 4-step Protocol (red, middle) and the Oasis PRiME MCX 3-step Protocol (green, right). Error bars show one standard deviation (n=8).

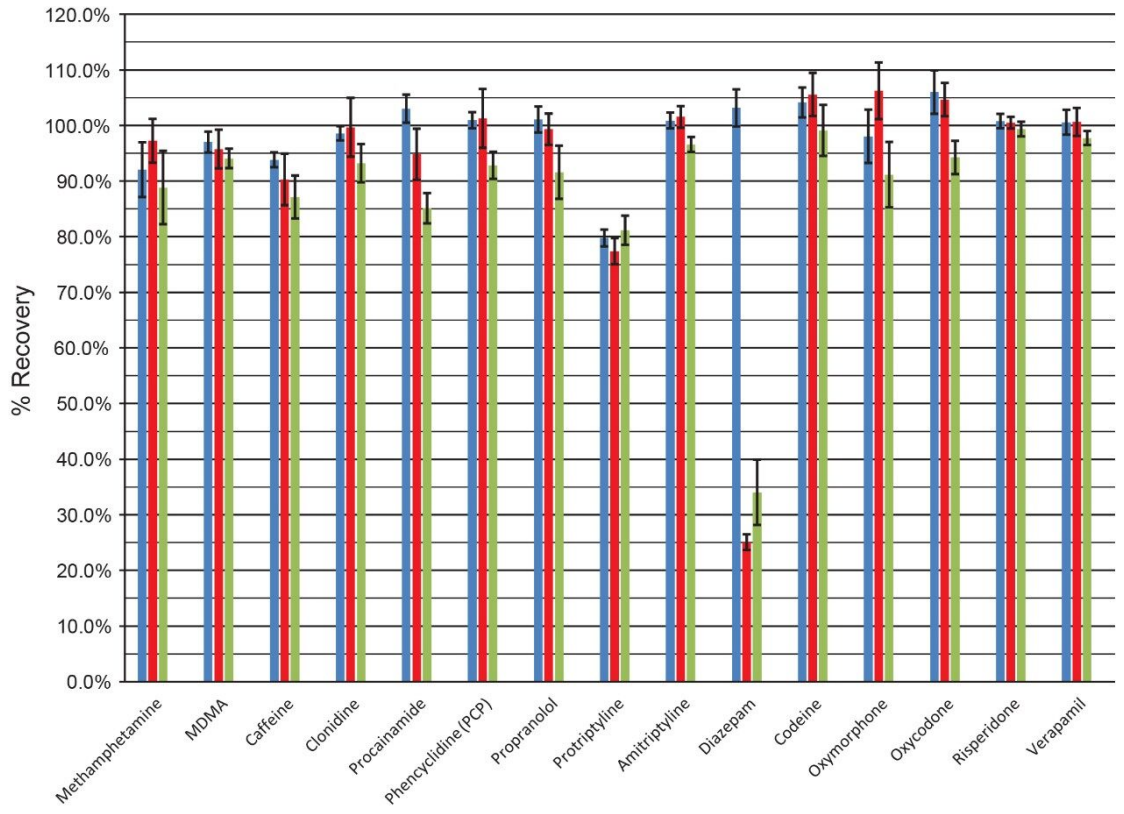

Figure 7. Elute step analyte recoveries for 15 basic compounds from rat plasma using the Oasis MCX 2 x 4 Protocol 1 (blue, left), the Oasis PRiME MCX 4-step Protocol (red, middle) and the Oasis PRiME MCX 3-step Protocol (green, right). Error bars show one standard deviation (n=4).

Extraction of basic analytes from plasma using the oasis prime MCX 3-step Protocol

A further simplification of the Oasis PRiME MCX 4-step Protocol is possible for aqueous samples. We found that when 100 mM ammonium formate is used in the Load step, the Aqueous Wash step can be eliminated without compromising the phospholipid removal or analyte recovery. The resulting Oasis PRiME MCX 3-step Protocol is described in Figure 5. This method was demonstrated for spiked rat plasma in Figure 6, using the same quantification strategies described in the previous section. The average phospholipid level is slightly higher than that obtained using the Oasis PRiME MCX 4-step Protocol, but still much lower than that observed for the Oasis MCX 2 x 4 Protocol 1. The analyte recoveries, shown in Figure 7, are similar to those achieved using the Oasis PRiME MCX 4-step Protocol. This Oasis PRiME MCX 3-step Protocol represents a considerable simplification. When hydrophilic interferences are present, both Oasis PRiME MCX protocols should be evaluated since the 3-step Protocol variant lacks the Aqueous Wash step.

Conclusion

This application note highlights improved SPE methods for extracting basic analytes from blood plasma using Oasis PRiME MCX sample preparation products. The new Oasis PRiME MCX Protocols give greatly reduced concentrations of phospholipids compared to the Oasis MCX 2 x 4 Protocol 1, while maintaining high and reproducible recoveries for analytes with pK_a values greater than 5. The lower phospholipid concentrations obtained with these new protocols are expected to result in reduced matrix effects in LC-MS applications, as well as less frequent issues with fouling of columns and LC-MS systems.

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